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EFFECTS OF TEMPERATURE EXTREMES ON PROTEIN SYNTHESIS IN LIVER OF TOADFISH, *OPSANUS TAU, IN VIVO*

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Abstract

Amino acid incorporation and polypeptide chain elongation rates were determined in toadfish at the upper and lower ends of their range of temperature tolerance. The method was based on pulse injection of radioactive amino acids into the hepatic portal vein and analysis of ribosome-bound and completed chains at various times after injection. Elongation rates at low temperatures were obtained from the rate of completion and release of polypeptide chains pre-labeled at 20°C. Average polypeptide chain assembly time at 4°C was 6 h; that at 37°C was 1 min. Comparison with rates of total protein synthesis obtained in previous studies indicate a coordinated slowing of all protein synthetic reactions at low temperatures. A 10-fold decline in elongation rate from 7°C to 4°C suggests a specific temperature sensitivity in elongation factors or in formation of aminoacyl-tRNA. At high temperatures (32°–40°C) protein synthetic reactions show a loss of coordination, with elongation rate increasing normally (Q_{10} about 2) while amino acid incorporation declines. The results indicate a close correlation between behavior of the protein synthetic system and observations on temperature tolerance and winter dormancy in this species.

Introduction

The toadfish is a highly adaptable marine fish that is common along the Atlantic coast from the Gulf of Maine to Cuba. Its broad temperature tolerance and hardiness in the laboratory have made it a valuable organism for examination of temperature effects on biological processes *in vivo*. Previous studies on liver protein synthesis in 20°C-acclimated toadfish *in vivo* have indicated three types of response to direct temperature change (Mathews and Haschemeyer, 1978). From about 17°C to 30°C, rates of polypeptide chain elongation and of total amino acid incorporation (measuring both initiation and elongation) show a normal Q_{10} of about 2.5. (Q_{10} is the ratio of rate at $T + 10$°C to that at T). From 17°C down to about 7°C, Q_{10} for both processes is approximately 5. With further temperature reduction, incorporation rate declines with an even greater Q_{10}, and elongation rate can no longer be measured conveniently.

The present work examines in more detail the behavior of this system at both low and high temperature extremes. An experimental method has been used that permits effects on polypeptide elongation reactions to be distinguished from those on chain initiation or ribosome recruitment. This is of particular interest because of the loss of coordination among these reactions observed at temperature extremes in all other organisms studied. In *E. coli*, for example, initiation of protein synthesis

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is strongly inhibited at temperatures below 8°C, whereas elongation continues at a relatively normal rate (Das and Goldstein, 1968; Friedman *et al.*, 1971). This causes a run-off of ribosomes from the messenger and consequent disaggregation of the protein synthetic apparatus. A similar phenomenon occurs at high temperatures (42°–43°C) in bacterial and mammalian systems (McCormick and Penman, 1969; Patterson and Gillespie, 1972; Oleinick, 1979).

Previous studies on elongation rate and ribosome run-off have depended on analysis of polyribosome profiles. This has not been found to be a reliable method for toadfish liver because of degradation by endogenous ribonuclease (Haschemeyer, 1969a). The direct method of labeling the growing polypeptide chains and following their rate of completion and release (Haschemeyer, 1969b) is not suitable when both the incorporation of radioactive amino acids and their uptake by the tissues are significantly reduced (Persell and Haschemeyer, 1980). We have therefore adopted the approach of pre-labeling the ribosome-bound polypeptide chains by hepatic portal vein injection at 20°C, then measuring the release of these chains after fish are cooled to the experimental temperature. For experiments at high temperatures the standard pulse injection method (Mathews and Haschemeyer, 1978) has been used.

Materials and Methods

Collection and maintenance of specimens

Opsanus tau adults, 300–400 g, were obtained from local collectors at the Marine Biological Laboratory, Woods Hole, Massachusetts, and were maintained during the summer in running seawater aquaria at 20° ± 1°C. Fish were fed chopped baitfish on alternate days; food was withheld for two days prior to experiment. For experiments at 4° ± 1°C, fish were kept in well-aerated 20 gallon aquaria in the coldroom. Fish acclimated to 10°C were kept in refrigerated running seawater aquaria for three weeks prior to use.

Experimental procedure at 20°C

Fish acclimated for at least two weeks at 20° ± 1°C were lightly anesthetized by brief exposure to 0.5 g/l ethyl-p-aminobenzoate (benzocaine) in well-aerated seawater at 20°C. The fish were transferred to a surgical rack and oxygenated seawater (without anesthetic) was circulated over the gills. The fish showed active operculation throughout the subsequent procedure. A small abdominal incision was made to expose the hepatic portal vein, and 0.1 ml of 15 m*M* (2 μCi) L-[^{14}C(U)]leucine and 2 μCi [^{3}H(G)]inulin (New England Nuclear Corp.) in balanced salts was slowly injected into the vein through a 30-ga needle (Haschemeyer, 1973). The presence of inulin served as a marker for the passage of the injection bolus through the liver (Persell and Haschemeyer, 1976). During injection, mixing of the injection solution with the portal blood was monitored visually.

For incubation times of 1 to 4 minutes, the needle was kept in the vein to prevent bleeding, after which the liver and blood draining from the liver at the hepatic vein were collected. For experiments of longer than 5 minutes duration the needle was removed, bleeding was stopped with gauze, and the abdominal incision was closed with wound clips. The fish was then quickly aroused by running 20°C seawater over the gills and returned to 20°C aquaria for a free-swimming period prior to collection of the liver. The procedure was standardized so that the time elapsed from the beginning of the injection until arousal was 5 ± 0.5 minutes.

Temperature transfer experiments

Fish acclimated at 20° ± 1°C were injected at 20°C and aroused after 5 minutes, as described above. They were immediately transferred to a container of seawater at 4°C and then to a 20 gallon aquarium in the coldroom. The rate of cooling was monitored with a rectal temperature probe connected to a Yellow Springs Telethermometer. Temperature was observed to decline with an approximately exponential dependency; cooling half-time (time required for a temperature change equal to one-half the final ΔT) was 5–7 minutes. All fish reached a temperature of 4°–5°C, as registered by the probe, within 30 minutes. Fish were then maintained at this temperature for varying periods up to 24 hours, after which liver and blood were collected.

Two groups of fish were subjected to a second temperature transfer as follows: After injection at 20°C and cooling to 4°C, as described above, they were maintained at 4°C for 1.5 to 16 hours, then returned to running seawater aquaria at 20°C. Livers were collected after 5 to 20 minutes exposure to the higher temperature. Another group of 20°-acclimated fish was cooled to 4°C without injection, maintained at that temperature for 24 hours, then returned to 20°C aquaria. After a 30 min equilibration period they were injected at 20°C for analysis of elongation rate and fractional incorporation at that temperature.

High temperature experiments

Fish acclimated at 20°C were adjusted to a temperature of 30°–32°C for 15 minutes and then anesthetized by brief exposure to 0.5 g/l benzocaine at that temperature. Each fish was further warmed on the surgical rack by a rapid circulation of heated seawater through the mouth and gills. An abdominal incision was made, and temperature was monitored with a thermometer placed under the liver. After a warming period of about 5 minutes, hepatic portal vein injection was performed as above except that a concentration of 0.1 m*M* ^{14}C-leucine was used. The liver was excised and hepatic blood was collected 30–60 seconds after injection.

Sample preparation and analysis

Livers were analyzed for levels of free ^{14}C and ^{3}H in the tissue and for incorporation of ^{14}C-leucine into protein (Haschemeyer and Persell, 1973). Blood collected from the severed hepatic vein was also analyzed for both isotopes. All data were converted to μCi per g liver or per ml plasma and divided by the μCi injected to yield fraction of dose recovered in the various compartments. Incorporation into protein is presented as total percent of injected ^{14}C-leucine recovered in protein-bound form or as fractional incorporation rate (fraction of dose incorporated divided by incubation time), as described by Mathews and Haschemeyer (1978). Uptake of ^{14}C-leucine into intracellular space was obtained from total free radioactivity in liver after subtraction of a small fraction associated with extracellular space. The latter was estimated by use of the inulin recovery in liver and the ^{14}C-amino acid/^{3}H-inulin ratio in hepatic blood plasma (Persell and Haschemeyer, 1976).

For determination of polypeptide chain assembly time incorporation into the total liver homogenate (T) was compared with that in the ribosome-free supernatant (S) obtained after centrifugation of the homogenate for two hours at 100,000 $\times g$. Homogenates were first treated with sodium deoxycholate at 0.5% to dissolve membranes. Radioactive incorporation was measured on filter discs (Haschemeyer, 1969b) in a toluene-based scintillation fluid at 70% efficiency.

Calculations

For determination of average polypeptide chain assembly time (t_c), the ratio of radioactivity in soluble protein (S) to total protein (T) of liver was plotted *vs.* time. The linear portion of the curve (up to S/T = 0.5) was analyzed to obtain t_c (Haschemeyer, 1969b). Elongation rate (residues per second) was calculated from t_c by use of the number-average molecular weight (45,000) of newly-synthesized polypeptide chains in toadfish liver, as follows:

$$\text{amino acid residues/sec} = (45{,}000/115)/60t_c \qquad (1)$$

where 115 is the average residue weight in protein (Mathews and Haschemeyer, 1978).

Results

Control experiments at 20°C

These experiments were run to test the validity of the modified system for determination of elongation rate *in vivo*. The method, as originally developed, depends upon the measurement of the distribution of labeled polypeptide chains between ribosomes (R) and the soluble compartment (S). The early linear dependency of S/T on time, where $T = R + S$, yields average polypeptide chain assembly time (Haschemeyer, 1969b). This may be combined with number-average molecular weight to obtain elongation rate in residues/sec (Eqn. 1). The time dependency, however, is influenced by the molecular weight distribution of chains synthesized; at later times weight-average molecular weight has a greater influence on S/T (Mathews and Haschemeyer, 1976). Use of these equations also requires a constant specific radioactivity during the experimental period. To better approximate this condition over the time periods required for temperature transfer, a higher concentration (15 mM) of the radioactive amino acid was used.

Figure 1 (left) presents the S/T results obtained in 20°-acclimated fish at 20°C using the 15 mM injection dose. The early linear portion yields a value of 5.5 min for t_c (average polypeptide chain assembly time), corresponding to an elongation rate from Eqn. (1) of 1.2 residues/sec. A similar experiment was run at 20° with

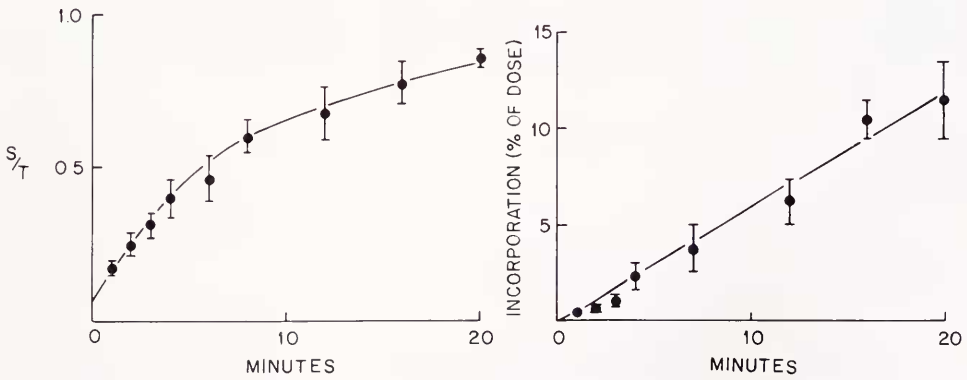

FIGURE 1. Protein synthetic parameters for toadfish liver *in vivo* at 20°C. Left: Ratio of incorporation into soluble protein (S) to total soluble protein + ribosome-bound chains (T) as a function of time after hepatic portal vein injection of 15 mM ^{14}C-leucine. Right: Incorporation into protein as % of injected dose. Animals were 20°C-acclimated toadfish, 370 ± 80 g body weight, % liver/body weight = 2.3 ± 0.5, number of fish = 30. Bars express standard deviation.

fish acclimated at 10°C for three weeks; t_c was 3.4 min, yielding an elongation rate of 1.9 residues/sec. Both values are in good agreement with results obtained at tracer or plasma leucine (0.1 mM) concentrations (Haschemeyer, 1969b; Mathews and Haschemeyer, 1978). At longer times S/T is observed to rise slowly toward 1.0. This behavior is consistent with theoretical prediction (Mathews and Haschemeyer, 1976) for a system in which continued incorporation of radioactivity into growing chains is occurring. Similar experiments with tracer doses yielded a more rapid rise of S/T to unity associated with a declining free radioactivity in the tissue.

The time course of incorporation of ^{14}C-leucine into protein at the 15 mM dose is shown on the right in Figure 1. The linearity over a period of 20 minutes indicates relatively constant specific radioactivity in the intracellular leucine pool. Incorporation rate in 20°-acclimated fish was 0.6% of the injected dose per minute; that in 10°-acclimated fish measured at 20°C was 1.2% per minute. Total recovery of radioactive leucine in liver (free + protein-bound) averaged 20% of dose; this is about one-half the level found after injection of 0.1 mM ^{14}C-leucine. Previous studies have indicated that declining fractional uptake with concentration is associated with saturation of the leucine transport system (Persell and Haschemeyer, 1976).

Polypeptide chain elongation after transfer to 4°C

The object of these experiments was to determine whether elongation of polypeptide chains continues in toadfish liver at very low temperatures. Previous work had indicated a large drop in incorporation of radioactive leucine into protein at temperatures below 7°C (Mathews and Haschemeyer, 1978). If this was due to a failure in initiation as in *E. coli* discussed above, ribosome run-off would occur leading to a reduction in concentration of active protein synthetic units. Conversely, the decline in protein synthesis might be due to a slowed rate of elongation without loss of active ribosomes. The time dependency of the system at 20°C described above indicated that it should be possible to label ribosome-bound polypeptide chains at 20°C and cool the animal before complete release of labeled chains had occurred.

Figure 2 presents the results for levels of incorporation of ^{14}C-leucine into protein, as % of dose, in fish labeled at 20°C and transferred to 4°C. These levels primarily reflect incorporation occurring during the 5 minute period at 20°C (about 2.5% of dose from Fig. 1) plus that taking place during cooling. Protein synthesis in this period would be expected to follow the $Q_{10} = 5$ dependency previously found for this temperature range (Mathews and Haschemeyer, 1978). The results at 0.75–

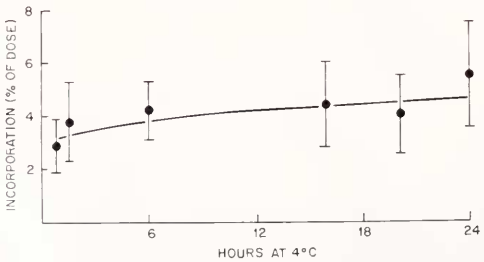

FIGURE 2. Incorporation levels in fish labeled at 20°C and transferred to 4°C. The recovery of ^{14}C-leucine in protein is expressed as % of dose administered. Bars indicate standard deviation; number of fish = 44.

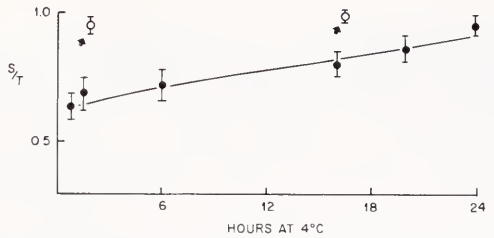

FIGURE 3. Time dependency of release of pre-labeled polypeptide chains at 4°C. The ratio of incorporation into soluble protein (S) to total soluble protein + ribosome-bound chains (T) is plotted as a function of time at 4°C. Closed circles: fish maintained at 4°C until analysis; open circles: fish re-warmed to 20°C after the indicated time at 4°C. Bars express standard deviation; N = 4–6 at each time point.

1.5 hours after injection (see Fig. 2) thus include both of these contributions. Further incorporation at 4°C is clearly very low (0.1% of dose per hour) and is obscured by individual variation. Such a low rate is consistent with data obtained when ^{14}C-leucine is injected directly at 4°C (Mathews and Haschemeyer, 1978). Measurements of free radioactivity indicate that ^{14}C-leucine is available for incorporation throughout this period, although declining from 20% of dose at t = 5 min to 7% at 24 h.

Figure 3 presents the S/T data for the fish transferred to 4°C. Individual variation here is much less than in Figure 2 because S/T is less dependent on amino acid uptake and specific radioactivity of the intracellular pool than are S and T separately. These data clearly show a continuous slow rise in S/T following the pre-labeling and cooling period. Most significantly, there is no evidence of the rapid approach to unity expected if elongation proceeds normally while initiation is inhibited.

In order to test the reversibility of the profound slowing of elongation indicated by the data of Figure 3, two groups of fish were transferred to 4°C and equilibrated for 1.5 or 16 h, then returned to 20°C water. The open circles in Figure 3 indicated by arrows represent average values of S/T found for these animals after 10–20 minutes of 20°C exposure. Values at 5 min were somewhat less. These results are consistent with resumption of elongation at a normal rate, leading to completion and release of the pre-labelled chains. The possibility that the protein synthetic system simply fell apart as a result of these temperature changes was not supported by the results in another control experiment. Fish were exposed to 4°C for 24 h without prior injection, re-warmed to 20°C as above, then analyzed in an experiment comparable to that of Figure 1. The data indicated normal protein synthetic behavior: incorporation rate was 0.75% of dose/min (±0.2%; N = 7); average polypeptide chain assembly time was 4.7 minutes. These results do not differ significantly from those in the fish not subjected to temperature change (Fig. 1).

The S/T data of Figure 3 may be analyzed by comparison with a theoretical curve where S/T is 0.5 after one round of protein synthesis, 0.75 after two rounds, and so on (Haschemeyer, 1969b). This, however, does not take into account heterogeneity in chain size which affects the S/T profile, particulary at $S/T > 0.5$ (Mathews and Haschemeyer, 1976). The problem is simplified by reference to the control data at 20°C (Fig. 1) where average chain assembly time is known. On this basis, the change in S/T profile observed during the time period of 4 to 24 hours at 4°C is equivalent to 3.2 rounds of protein synthesis. This yields an average chain assembly time at 4°C of 6 hours; elongation rate is 0.018 residue/second.

Effects of elevated temperatures

These experiments were designed to test the ability of the liver protein synthetic system to maintain coordination during acute exposure to high temperatures. Fish acclimated at 20°C were first brought to 30°C for about 15 min, then rapidly warmed with heated seawater to temperatures ranging from 32°C to 40°C. The speed of protein synthesis was such that it was necessary to reduce incubation times to 30–60 sec in order to stay within the linear range of S/T. The S/T results yielded continuously increasing values for elongation rate with temperature (Q_{10} about 2), as shown in Figure 4. This behavior is similar to that observed in mammalian cells in culture, although the possibility of premature termination of chains under these conditions cannot be ruled out. Average polypeptide chain assembly time at 37°C was 1 minute, as in rat liver (Mathews and Haschemeyer, 1976).

Fractional incorporation, reflecting both elongation rate and active ribosome concentration, however, declined rapidly at temperatures above 32°C. The Arrhenius plot (Fig. 4, closed circles) indicated a Q_{10} of about 1/40 or activation energy of −290 kJ/mole. This fall in incorporation did not appear to be a consequence of reduced uptake of the radioactive amino acid. Levels of intracellular ^{14}C-

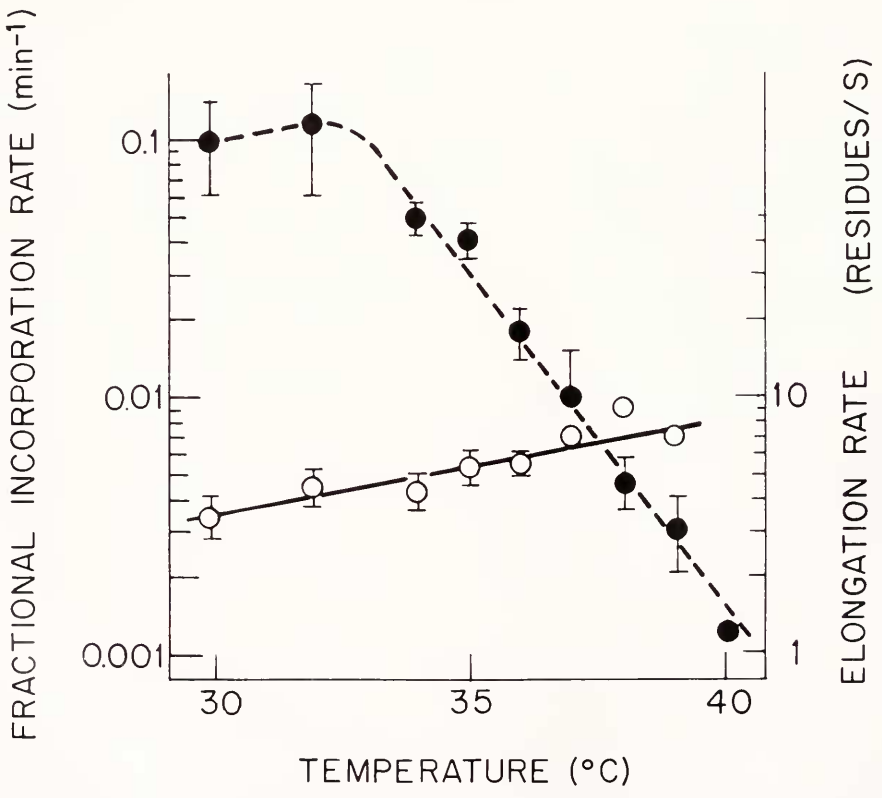

FIGURE 4. Arrhenius plot for toadfish liver protein synthesis at high temperatures. The fractional rate of incorporation of ^{14}C-leucine into protein (closed circles, left scale) and elongation rate (open circles, right scale) are given as a function of body temperature. Exposure time at each temperature was about 5 minutes. Rates are given on a logarithmic scale; bars indicate standard deviation; number of fish = 30.

leucine at 1 min after injection averaged 25% of dose from 30° to 38°C, close to levels observed at more moderate temperatures (Persell and Haschemeyer, 1976). Above 38°C, uptake fell to 15% of dose, suggestive of membrane dysfunction.

Fish that were warmed to a body temperature of 37°C, then returned to 30°C, showed normal protein synthetic parameters at 30°C. Fish exposed to higher temperatures (up to 43°C) exhibited only partial recovery (10–25%) of incorporation rate when returned to 30°C. Effects on the central nervous system also were evident at the higher temperatures. Partial jaw clamping occurred at 36°–38°C; tight clamping and body stiffening were observed at 39°–43°C. However, all fish maintained operculation and gave evidence of normal circulation, based on inulin movement through the liver. None died during the short periods of these experiments, although tests using one-hour exposures indicated a lethal temperature of about 35°C.

Discussion

The experiments presented above represent the first detailed analysis of protein synthetic parameters at extreme temperatures in a vertebrate liver *in vivo*. Previous attempts to characterize this system in toadfish at very low temperatures were limited by the low levels of uptake of radioactive amino acids and of incorporation into protein (Mathews and Haschemeyer, 1978). The method presented here permits determination of elongation rate from the rate of completion of pre-labeled polypeptide chains. Hepatic portal vein injection was used to obtain rapid uptake and labeling of chains. A more constant specific radioactivity was achieved by the use of 15 m*M* leucine in place of the usual tracer or plasma concentration. This helps to minimize differences in pool size as well as time-dependent changes in specific radioactivity caused by turnover. Pool swamping has been used effectively for measurements of protein synthesis in mammals (see, *e.g.*, McNurlan and Garlick, 1980). Studies in toadfish indicate that the 15 m*M* leucine injection does not affect liver protein synthesis as measured by incorporation of another amino acid (Persell and Haschemeyer, 1976).

The results of the temperature transfer experiments indicate that elongation of chains proceeds very slowly at 4°C, at a rate of about 1/10th that previously observed at 7°C. The effect accounts for the observed change in total protein synthesis in this temperature range, as measured by direct amino acid incorporation (Mathews and Haschemeyer, 1978). There is thus no indication of a specific blockage of initiation leading to ribosome run-off in this system. Rather, the response is evidently a slowing down of all steps without loss of coordination among chain initiation, elongation, and release reactions. Under these conditions the system is able to shift up immediately in response to increased temperature, as evidenced by the re-warming experiments.

The properties described above are consistent with the ability of the toadfish to adapt to widely fluctuating environmental temperatures and with its dormancy in winter (Gudger, 1908). The molecular basis for the large depression in elongation rate, however, is not clear. Measurements of aminoacyl-tRNA binding by elongation factor 1 do not give evidence of any unusual temperature sensitivity (Plant *et al.*, 1977). The synthesis of the precursor aminoacyl-tRNA, however, could be affected, and is currently under study. Protein synthetic rates may also be influenced by membrane transitions in this temperature range. Another factor that may play a role in the toadfish system is the requirement for adjustment of blood pH with temperature change (Rahn and Baumgardner, 1972).

The behavior of the toadfish system at temperatures approaching the upper lethal limit resembles that seen in other organisms. Total protein synthesis reached its maximal rate at 32°C, then deteriorated rapidly as temperature was increased. Elongation and release of chains, however, showed an apparently normal increase in rate up to 38°C. This would support a specific effect on initiation reactions, as suggested for other systems (McCormick and Penman, 1969; Patterson and Gillespie, 1972; Oleinick, 1979). A failure of initiation complex formation occurs *in vitro* at the same temperatures found to inhibit protein synthesis in whole cells (Mizuno, 1975; Bonanou-Tzedaki *et al.*, 1978). These temperatures, however, approach the upper limit of membrane integrity (see, *e.g.* , Janoff *et al.*, 1979, for *E. coli*). In the toadfish system the changes in protein synthesis were observed at temperatures well below those at which ^{14}C-leucine uptake by liver was affected. The activation energy for inhibition was similar to that observed for protein denaturation (Johnson *et al.*, 1974). Although not conclusive, these results would support the view that denaturation of a specific protein synthetic component may be a primary event, as opposed to general membrane dysfunction. Further studies in isolated cells may be helpful in distinguishing these effects. Also of interest is the possibility that disassembly of the protein synthetic system at high temperatures is associated with a shift to production of heat shock proteins, as in other organisms (Lemaux *et al.*, 1978; Moran *et al.*, 1978).

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